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Interferons (IFNs) and retinoic acid (RA) are potent biological response modifiers that inhibit transformed cell growth. In tumor cells resistant to either IFN or RA, combination of both agents causes stronger growth inhibition. Using athymic nude mouse models and in vitro systems we have shown earlier that IFNβ/RA combination causes cell death of human breast carcinoma cells. Since we could not find a correlation between expression of known regulators and cell death, we employed the antisense technical knock-out strategy to isolate genes that participate in IFN/RA induced pathways. We prepared total cell cDNA libraries from breast tumor cells treated with IFN/RA combination and cloned the inserts in antisense orientation into the episomal vector pTKO1. Following transfection of these libraries the breast tumor cells were selected for resistance to IFN/RA induced cell death. Using this technique we have isolated several candidate genes termed **G**enes associated with **R**etinoid-IFN **I**nduced **M**ortality (GRIM). In this report, we have identified one of these genes, GRIM-12. Sequence analysis revealed that GRIM-12 is identical to human thioredoxin reductase (TR). IFN/RA combination stimulates the post-transcriptional expression of GRIM-12. The enzymatic activity of TR was essential for mediating cell death. Dominant negative mutants have been made to characterize this gene further. Another gene, GRIM-1, has been identified as a novel sequence. Northern analysis demonstrated the IFN/RA combination induces two GRIM-1transcripts. cDNA λ phage libraries were screened in attempt to clone the full length cDNA.

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Edward P. Thomann 7/28/98

PI - Signature Date

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Introduction:

This training fellowship was awarded to a pre-doctoral student, Edward R. Hofmann, at the University of Maryland School of Medicine. Research data generated from this study will be submitted as a Ph. D. thesis in the future.

Interferons (IFN) are a group of multi functional cytokines which regulate cellular antiviral, antitumor and immunological responses (1, 2). Products of IFN-stimulated genes (ISGs) mediate the biological actions of IFNs (2). IFNs use the JAK/STAT signal cascade to regulate ISGs (3, 4). Despite the description of several cellular ISGs, the molecular mechanisms of growth inhibition by IFNs are unknown. Among the known gene products two antiviral pathways have been suggested to mediate growth inhibition. These are: 1) the 2-5A pathway and 2) the PKR (protein kinase R) pathway (1, 5, 6). Both these pathways are operated by double stranded RNA-dependent enzymes. However, we have not found evidence for the involvement of these pathways in IFN + RA mediated growth inhibition. Although IFNs as single agents have strong anti-tumor activities in a variety cancer cells, they have limited effects on breast tumor cell growth (7, 8). Therefore, we explored whether a combination of IFNs with retinoids would cause strong growth inhibition in breast cancer cells.

Retinoids encompass vitamin A related compounds which have profound influences on cell growth and differentiation (9). A prototype of these is all-trans retinoic acid (RA), a natural metabolite. Retinoids interact with specific nuclear retinoic acid receptors (RAR) which then associate with cognate retinoic acid response elements (RARE) to stimulate gene expression (9, 10). Retinoids suppress the growth of several tumor cells *in vitro* and *in vivo* (11). Despite their strong cell growth regulatory action, the candidate genes for retinoid mediated growth inhibition have been unknown. Although many studies have suggested a role for RARs in growth inhibition of RA (12, 13), these data so far have not identified the ultimate gene product(s) that mediate the growth inhibition. Furthermore, our laboratory and others have found that RA by itself is not sufficient for breast tumor growth inhibition *in vitro* and *in vivo* (14).

Previous studies in our lab have demonstrated that IFN-β and RA alone are unable to inhibit cell growth in several breast tumor cell lines. However, IFN-β + RA combination induces growth arrest of several breast carcinoma cells both ***in vitro*** and ***in vivo*** (15). Furthermore, high concentrations or extended exposure to IFN-β + RA induces a cell death consistent with programmed cell death. Preliminary studies identified no changes in the levels of known regulators of cell death such as p53, cyclin D and Bcl2 (16-18). Thus it is important to understand the cell growth suppressive mechanisms and the genes that mediate these effects. We hypothesized that the observed cell death might be mediated by either novel or hither to unimplicated gene products. Therefore, the major aim of this project is to identify the gene products that mediate the growth inhibitory/ cell death inducing activities of the combination of IFN and RA in human tumor cells.

We have previously reported that utilizing an antisense knockout strategy (19, 20) we have isolated several independent candidate genes. In this method, cDNA libraries, prepared from BT-20 human breast carcinoma cells, and cloned into an episomal vector in an antisense orientation, were transfected into cells. The transfected cells were then treated with death inducing concentrations of IFN/RA combination and surviving clones were collected and expanded. The episomes were rescued from surviving clones and transformed into *E. coli* to isolate individual candidate genes. False positives were eliminated by re-transfecting breast tumor cells with individual episomes for second and third round screenings. These genes have been named

Genes associated with Retinoid-IFN Induced Mortality (GRIM). Two clones GRIM-1 and GRIM-12 have been chosen for further study. It was previously reported that partial sequence analysis of GRIM-1 showed no homology to any known gene sequences found in GenBank.

Body:

Experimental Approach:

GRIM-1 and GRIM-12 were chosen because of their large insert size and therefore greater chance of finding a complete cDNA. In order to characterize these clones we first will determine if they are expressed in breast carcinoma cell lines via northern analysis. From these data we will know the size(s) of the endogenous mRNA message and determine if we have the complete cDNA. Those clones smaller than the endogenous mRNA will then be used to probe a BT-20 cDNA library constructed in a λ phage, Uni-ZAP XR vector. This will allow us to screen a larger number of clones than our antisense library due to the greater efficiency of phage infection verses plasmid transformation. After obtaining a full-length insert, the clone will be sequenced to establish a complete cDNA map. Sequence analysis will be done to predict protein size and structure using a GCG program as well as DNA and protein databases such as GenBank and Swissprot. After determining the open reading frames of GRIM-1 and GRIM-12 we will express in vitro using rabbit reticulate lysates. Protein will also be expressed in a pET32 histidine-tag bacterial expression system to allow purification of protein expressed in *E. Coli.* These proteins can then be used for antibody preparation. Antibodies will allow us to determine expression and post-transcriptional regulation of the gene products. Sequence analysis will enable us to determine functional domains for mutational studies.

Materials and Methods:

Reagents: Restriction and DNA modifying enzymes (NE Biolabs); DTT, PMSF, Triton-X, Imidazole, RA (Sigma); G418 Sulfate, IPTG (Life Technologies); Ni-chelation-sepharose (Novagen); Glutathione Sepharose (Pharmacia Biotech); Nitrocellulose membranes, ECL reagents and horseradish peroxidase coupled to anti-rabbit antibodies (Amersham); human IFN-β_{ser} (Berlex Inc.), and Hygromycin B (Boehringer-Mannheim) were employed in these studies. Fresh stocks of RA were prepared in ethanol and added to cultures under subdued light.

Cell culture: All estrogen dependent cells were cultured in phenol red free EMEM supplemented with 5% charcoal stripped fetal bovine serum (CSFBS) and 10^{-11}M estradiol during treatment with IFN-β and all trans retinoic acid (RA). Cells were grown in phenol red free media 24h before treatments were initiated. All other cell lines were cultured in media with phenol red but supplemented with 5%CSFBS prior to treatment with IFN-β and RA.

Construction of Dominant Negative Mutants: Dominant negative mutants were constructed by PCR using the following primers: FAD (FAD binding active domain) was generated with 5'-CCAAGCTT**ATG**AACGGCCCT GAAGATC as the forward primer and 5'-CCGAATTC**TCA** TAAGCATTCTCATAGACGAC-3' as the reverse primer. NBD (NADPH binding domain) was prepared with 5'-CCAAGCTT**ATG**CAATTTATTGGTCCT CACAG-3' as the forward primer

and 5'-CCGAATTC**TCA**TGTCTTATAAACTTGATGCC-3' as the reverse primer. The ID (Interface domain) was generated by using 5'-CCAAGCTT**ATG**GTGCCTTACATC TATGCC-3' as the forward primer and 5'-CCGAATTC**TCA**GCAGCCAGCCTGGAG-3' as the reverse primer. Respective start and stop codons are shown in bold. PCR products were digested and cloned into the pCXN2 mammalian expression vector, in which the chicken actin promoter regulated the expression of mutants. It also carried a neomycin resistance marker for selection in mammalian cells. Stable transfectants were generated by electroporation of cells with indicated plasmids and selection with G418 (500 μg/ml). After three weeks of selection, surviving clones were expanded for further studies. Individual mutants were designated as FAD, NBD, or ID. Expression of mutants was monitored by northern blot analysis.

Northern Blot Analysis: Total RNA (20μg – 40μg) was separated on 1% formaldehyde agarose gels, transferred to a nylon membrane, and probed with ^{32}P labeled PCR product of GRIM-12 and GRIM-1 cDNAs. Prehybridization, hybridization and washing was carried out under stringent conditions (22).

Western Blot Analysis: Total protein was extracted after various treatments using the freeze/thaw method. Total protein (10μg) was separated on a 10% acrylamide gel and transferred to PVDF membrane (NEN). Membranes were incubated with a primary antibody raised against the peptide VVGFHVLGPNAGEVTQGFAA, derived from the native enzyme (23). After stringent washing, membranes were incubated with anti-rabbit IgG antibody conjugated to horseradish peroxidase and developed using ECL reagents (Amersham).

***In Vitro* Transcription and Translation:** GRIM-12 was subcloned into pBluescript KS vector (Stratagene) under the control of T7 promoter. Plasmid DNA (1μg) was linearized with HindIII and *in vitro* transcription was performed from the T7 RNA polymerase (Promega). The resultant RNA was programmed into nuclease treated rabbit reticulocyte lysates (Promega) in the presence of ^{35}S-methionine. Translation products were separated on 10% SDS-PAGE, dried and fluorographed.

Bacterial expression: pET-32B (Novagen) and pGEX-2TK (Pharmacia Biotech) were used as cloning vectors. GRIM-12 ORF was PCR amplified from pTKO1-GRIM-12 cDNA clone using a forward primer : 5'-CCAAGCTT**ATG**AACGGCCCTGAAGATC-3' and a reverse primer 5'-CCGAATTC**TCA**GCAGCCAGCCTGGAG-3'. These primers contained Hind III and EcoRI sites, respectively, for directional cloning. PCR was carried out for 10 cycles. PCR product was treated with T4 DNA polymerase and digested with Hind III. It was subcloned into pET-32B at Hind III and Xho I (site was blunt ended). ID was PCR amplified from pTKO1-GRIM-12 cDNA clone DNA using a forward primer: 5' CCAAGCTT**ATG**GTGCCCTACATCTATGCC 3', and a reverse primer: 5' CCGAATTC**TCA**GCAGCCAGCCTGGAG 3'. These oligonucleotides contained BamHI and EcoRI restriction sites, respectively for sub cloning the amplified inserts. The PCR product (535 bp) was digested with EcoRI and BamHI, and subcloned into pGEX-2TK.

pET-32B-GRIM-12 and PGEX-2TK-ID plasmids were transformed into *E.coli* BL21DE3 and transformants were grown in 2YT medium. A 2 liter culture was induced with IPTG (0.1 mM) at midlog phase for 4h at 37°C. Cells were harvested, washed with 200 ml of buffer (20

mM Tris-HCl, pH 7.9, 500mM NaCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 0.01% Triton-X and 5mM DTT) and suspended in 20 ml of buffer. Cells were sonicated and the clarified supernatants were passed through Ni-chelation-sepharose (Novagen) or glutathione Sepharose 4B (Pharmacia Biotech) depending on the fusion tag as recommended by manufacturer. After elution, proteins were separated on 10 % SDS-PAGE gels, followed by silver staining.

Enzymatic assay: Thioredoxin reductase activity was determined as described (24). Cell extracts were prepared after IFN/RA treatment by freeze-thaw lysis. Twenty micrograms of extract was incubated with insulin, NADPH and thioredoxin in 0.2M HEPES, pH7.6 for 20 min at 37°C. Reactions were stopped with 6M guanidinium hydrochloride/ 0.4mg/ml dithiobis(2-nitrobenzoic acid) in 0.2M Tris, pH 8.0. Absorbance at 412 nm was measured. In each case a corresponding control without Trx was used to determine the basal level of TR activity (due to endogenous Trx and NADPH). Absorbance values obtained from these controls were subtracted from those obtained with the reactions that contained Trx and NADPH. A control reaction without cell extracts but with all the reaction components was also employed. Triplicate samples were measured for enzymatic activity. Pure TR was used as a positive control.

Isolation of GRIM-1 cDNA by λ phage library screening : BT-20 cells were treated with IFN-β (500U/ml) + RA (1μM) for 0, 2, 4, 8, 16, 24, 48 and 72 h and total RNA was prepared as described previously. cDNA was synthesized using the ZAP-cDNA Synthesis Kit (Stratagene) and packaged using the Gigapack III Gold phage packaging extracts (Stratagene). The library was grown once and used for isolation of specific genes. ^{32}P labeled cDNA restricted from pTKO-GRIM-1 clones were used to screen approximately $2X10^6$ pfu. Approximately 10 clones were identified by PCR. Two clones, S3P1#1and S3P7#13, were chosen for further study.

Results

Identification of GRIM-12 as human thioredoxin reductase: The GRIM-12 episome contains an insert size of 3536 bases. This insert was completely sequenced on both strands. The cDNA has a 155 nt long 5' untranslated region (UTR) and a 1896 nt long 3' UTR. Sequence analysis indicated a large open reading frame(ORF) encoding a polypeptide 495 amino acids long. The a predicted protein has a molecular weight of 54.4 kDa. The predicted amino acid sequence is shown in figure 1. Comparisons of GRIM-12 cDNA with DNA database sequences revealed that GRIM-12 is identical to thioredoxin reductase (TR)(23), except for 3 amino acid substitutions. These are an arginine in place of serine at 156, an asparigine in place of glycine at 215, and an arginine in place of serine at 491. The active site is formed by the two cysteine residues present at positions 57 and 62. Homology searches also revealed that in addition to TR, it also is identical to a protein described as "KM-102 bone marrow derived reductase like factor" (GenBank accession no. D88687), between residues 53-549(25). However, the KM-102 reductase has an additional 52 aa unique to it. Deduced protein sequence also exhibited a close homology to a predicted glutathione reductase gene from *Caenorhabitis elegans*. The sequence has been deposited in the GenBank. Accession number for this sequence is: AF077367

To examine whether the GRIM-12 cDNA contained an intact ORF, the insert was cloned into pBluescript under the control of the T7 promoter. The cloned insert was transcribed and translated *in vitro* using rabbit reticulocyte lysates. The translated product of GRIM-12 cDNA

encoded a protein that migrates as a 58 kDa species on 10% SDS-PAGE (Fig 2A). In order to express GRIM-12 in E. coli, we placed the ORF down stream of a histidine-tag in pET32B vector. The fusion protein was purified on Ni-chelation columns. SDS-PAGE analysis revealed the expression of a 78kDa fusion-protein (Fig 2B). This protein contained 20 kDa of tag-derived sequences. Removal of the tag yielded a 58 kDa protein (data not shown). Thus, in both eukaryotic and bacterial expression systems it encodes a polypeptide of 58 kDa, which was higher than the predicted 54.7 kDa. This difference could be due to post-translational modifications. Indeed, sequence analyses suggested several phosphorylation sites. Western blots were done on bacterially expressed fusion protein using rabbit polyclonal antibodies specific for human TR. These antibodies recognized the fusion protein but not the tag (Fig 2C). Thus, GRIM-12 encoded human TR.

Effect of IFN and RA combination on the expression of GRIM-12: Northern analysis was performed to study the effect of IFN/RA combination on GRIM-12 mRNA levels. GRIM-12 cDNA hybridized to a single endogenous 3.8 kb mRNA transcript in MCF-7 and BT-20 cells. There was no significant induction of GRIM-12 mRNA in cells treated with IFN/RA combination, however, there may be a slight increase at 48 and 72 h (Fig 3). Reprobing of these blots with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe confirmed the presence of comparable amounts of RNA in all the lanes (data not shown). Thus, transcription of GRIM-12 is not regulated by IFN/RA combination.

To explore the possibility that GRIM-12 could be post-transcriptionally regulated, we performed western blots on cells treated with IFN/RA combination. GRIM-12 protein was induced by IFN/RA combination in a time dependent manner. There was no detectable induction until 8 h post-treatment and protein levels increased progressively until 72 h (Fig 4). Enzyme activity was measured to see if there was a correlation between protein levels and TR activity. Insulin reduction assays were done to measure TR activity in cell extracts from cells treated with IFN/RA combination. Consistent with the increase in protein levels, TR activity increased progressively with the length of IFN/RA exposure to cells (Fig 5).

Antisense inhibition of TR expression by GRIM-12: In order to determine whether cells treated with IFN/RA were dying due to an increase in TR expression, we determined the levels of TR protein and enzyme activity, in cells transfected with the GRIM-12 antisense episome. To do this we used two stably selected pools of HeLa cells, one transfected with the pTKO1 vector as a control and one that expresses GRIM-12 antisense mRNA. Northern blots were performed to confirm that GRIM-12 antisense transcripts were expressed in GRIM-12 transfected cells (Fig 6A). When western blots were done on extracts from these cells, GRIM-12 transfected cells showed a strong reduction in TR protein (Fig 6B). Likewise, the same extracts showed a 7-fold reduction in enzyme activity compared to control (Fig 6C). These data indicate that antisense expression Of GRIM-12 in long-term culture can repress TR expression and activity. Furthermore, this suggests that repression of TR confers a growth advantage in those cells expressing antisene GRIM-12 transcripts.

Over expression of GRIM-12 mutants: To further define the role of GRIM-12 in cell death pathways, we next decided to over express specific subdomains of TR. The hypothesis to be tested is: whether the subdomains of GRIM-12 would act as dominant negative inhibitors. If so,

would they interfere with IFN/RA induced cell death. Based on the homology to the glutathione reductase-thioredoxin reductase family, GRIM-12 can be divided into three major functional domains(23). These domains consist of an N-terminal flavin adenine dinucleotide (FAD) binding domain (FAD domain or active domain), a central NADPH binding domain (NBD) and a C-terminal interface domain (ID). The FAD domain contains the cysteine residues essential for the reduction of its substrate, thioredoxin. The NBD domain binds nicotinamide adenine dinucleotide phosphate dehydrogenase (NAPDH), an essential cofactor for redox enzymes. The interface domain is believed to be important for dimer formation.

These domains were expressed as deletion mutants to see if they could act as dominant negative inhibitors of endogenous GRIM-12. Mutants were created by inserting PCR generated regions, representing each domain, into the eukaryotic expression vector, pCXN2. This vector contains a chicken β-actin promoter to drive expression of the insert and a neomycin resistant cassette for stable selection in mammalian cells. Stable transfectants were made expressing these domains in MCF-7 and BT-20 cell lines. Figure 7 shows a northern blot demonstrating expression of comparable amounts of transcripts for each domain. Controls cells transfected with vector alone did not show expression of any transcripts other than endogenous TR. Growth rates of stable cell lines did not appear to be different for various mutants (data not shown). We have just obtained these cell lines and we will be testing the effect of IFN/RA combination on these cells.

IFN and RA combination induces two related mRNA species of GRIM-1: Northern analysis was performed on mRNA from MCF-7 and BT-20 breast cell lines treated with IFN/RA combination. In both cell lines, a GRIM-1 probe hybridized to two similar length mRNA species of 2.6 and 2.8 kilobases (Fig. 8). Both species are inducible in each cell line. The kinetics of induction are different for each cell line. MCF-7 mRNA induced as early as 24 h (Fig. 9B) while BT-20 mRNA is induced later at 48 h (Fig. 9A). This correlates to the observation that death in MCF-7 cells appears to occur slightly faster than BT-20 cells when treated with IFN/RA combination. Induction of mRNA was determined indirectly by probing the same blot with a GAPDH probe (Fig. 9A). Further analysis will be done to confirm this. MCF-7 mRNA blots showed comparable amounts of mRNA when probed with a GAPDH probe (Data not shown).

Cloning of GRIM-1 full length cDNA and identification of GRIM-1 as a novel sequence: Since northern analysis revealed two mRNA species of larger sizes than the 1912 base pair insert of GRIM-1 we have at hand, we screened a BT-20 cDNA library that was cloned into the Uni-ZAP XR vector and packaged into λ phage packaging extract. This allowed screening of a large number of clones (~$2x10^6$pfu). GRIM-1 cDNA insert was used as a probe. A total of 5 clones were picked for analysis. PCR revealed that two clones, S3P1#1 and S3P7#13, contained the largest inserts of 2.2 kb. These were chosen for sequence analysis. This revealed that both were identical in both size and sequence. In addition, overlapping regions were identical to GRIM-1. Even though we had obtained more than 300 bp of new sequence, we were about 400 bp short of the mRNA detected by northerns. Furthermore, the largest potential ORF was still incomplete. This cDNA is a novel one.

Conclusions:

Two antisense clones that have been isolated by use of a technical knockout strategy, have been chosen for further study. We have demonstrated that GRIM-12 is identical to human thioredoxin reductase. We have shown that our clone expresses a 58 kDa protein using both eukaryotic and prokaryotic systems. Although GRIM-12 is not transcriptionally regulated by IFN/RA combination, protein levels increase as early as 8 h post-treatment. This increase in protein levels correlates to an increase in enzyme activity. We have confirmed that expression of the antisense episome was responsible for a decrease in TR expression. This correlates to a decrease in TR activity in these cells.

We have also begun to characterize a novel gene, GRIM-1. Northern analysis demonstrated that GRIM-1 hybridizes to two similar size mRNA transcripts. These transcripts are inducible by treatment with the IFN/RA combination in both BT-20 and MCF-7 cell lines. Sequence analysis to date revealed that this cDNA represents a novel gene. Once the ORF has been confirmed more extensive structural and homology studies can be done.

Studies planned for the next budget year:

1. We will examine the dominant negative effects of the different domains of GRIM-12 cDNA. We have already established cell lines necessary for this study. We will determine enzyme activity and compare it death inducing effects of IFN/RA combination. Since the biologically active glutathione reductase is a homodimer(23), we predict that the interface domain (ID) will act as a dominant negative inhibitor of enzyme activity and cell death. However, other domains may also have dominant negative effects.

2. In order to determine if GRIM-12 function is impaired by the ID mutant disrupting dimerization, we will examine if this mutant is able to bind to GRIM-12 protein *in vitro*. We also will determine how specific GRIM-12 mediates IFN/RA death by treating cells expressing the deletion mutants with other known inducers of cell death (such as TNF-α) and measuring inhibition of cell growth. Finally we will see what effect overexpression of GRIM-12 has on cell growth.

3. We will over express the wild type GRIM-12 cDNA in MCF-7 breast carcinoma cells and determine whether it alone cause cell death or affects of growth cells. We will determine the effects of IFN/RA combination on the cells over expressing GRIM-12. A vector transfected control will be used for these studies.

4. We will confirm the ORF of GRIM-1 by use of eukaryotic and bacterial expression systems. Confirmation of the ORF will allow us to proceed with more extensive structural and homology studies. Mutational studies will give more insight into the functional domains of GRIM-1 as did the work done on GRIM-12. Expression in bacterial pET vectors will allow us to purify our protein for antibody production. Proteins involved in cell death sometimes are toxic to bacteria. If this is the case we will express deletion mutants to use for antibody production. If we are still unsuccessful, we will use synthetic peptides.

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Appendices:

Legends to Figures :

Figure 1: Amino acid sequence of GRIM12. Cysteine residues of the active site (underlined) are shown in bold.

Figure 2: Identification of GRIM-12 as human thioredoxin reductase. (A) GRIM12 cDNA cloned in pBluescript (pBS) vector was transcribed and translated in vitro using rabbit reticulocyte lysate. Protein encoded by GRIM-12 was indicated with an arrow. (B) Expression of GRIM-12 in bacteria. Bacterial lysates were purified on Ni-chelation sepharose column prior to SDS-polyacrylamide electrophoresis and silver staining. (C) Western blot analysis of the protein samples shown in Panel B, using a human TR specific antibody.

Figure 3: Northern blot analysis of GRIM-12 using RNA (20 μg) derived from MCF-7 and BT-20 cells after treatment with IFN-β (500U/ml) + RA (1μM) combination.

Figure 4: Western blot analysis of cell extracts (70 μg) prepared after IFN-β (500U/ml) and RA (1 μM) treatment as in figure 7. Blots were probed with TR specific antibody.

Figure 5: Thioredoxin reductase activity in IFN/RA stimulated MCF-7 cell extracts. Cells were treated as described in Figure 8. TR activity was determined using 20 μg of cell extract from each sample. Each bar represents the mean absorbance ± SE of triplicate measurements.

Figure 6: Antisense expression of GRIM-12 interferes with TR expression. HeLa cells transfected with pTKO1 or GRIM-12 episome were stably selected with Hygromycin B as described in materials and methods for 4 weeks. (A) Total RNA (40 μg) from each cell line was used for northern blotting. Blots were probed with a ^{32}P-labeled GRIM-12. Filled and open arrows indicate the positions of antisense and sense GRIM-12 mRNAs, respectively. (B) Expression of TR protein in the antisense GRIM-12 transfected cells. Equal amount of the cell extract (50 μg) from indicated cells was analyzed by western blots. Note the differences in expression of TR protein between the two cell types. (C) TR activity of the cells used in panels A and B. Cell extracts (20 μg) were assayed for TR as described under figure 9. Each bar represents mean absorbance ± SE of triplicates.

Figure 7: Expression of various functional domains of GRIM-12 (TR) in MCF-7 cells. Panel (A) shows the proposed functional domains of GRIM-12 protein and their approximate sizes. N and C indicate the amino- and carboxyl termini, respectively, of the native protein. Panel (B) shows the northern blot analysis of total RNA (40 μg) from various cell lines with GRIM-12 probe. Labels above the panel indicate plasmids transfected into the cells. V: pCXN2 vector; FAD: FAD domain, NBD: NADPH binding domain; ID: interface domain. FL: full length mRNA; DN: dominant negative mutants. Each lane had comparable amount of RNA as detected by GAPDH probe (not shown).

Figure 8: Northern blot analysis of GRIM-1 using total RNA (40μg) from (A) BT-20 and (B) MCF-7 cells after treatment with IFN-β (500U/ml) and RA (μM) combination.

MNGPEDLPKSYDYDLIIIGGGSGGLAAAKEAAQYGKKVMVLDFVTPTPLGTRWGLGGTCVNVGCIPKKLMHQAALLG
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ERFLIATGERPRYLGIPGDKEYCISSDDLFSLPYCPGKTLVVGASYVALECAGFLAGIGLNVTVMVRSILLRGFDQD
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SEEKAVEKFGEENIEVYHSYFWPLEWTIPSRDNNKCYAKIICNTKDNERVVGFHVLGPNAGEVTQGFAAALKCGLTK
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Figure 1

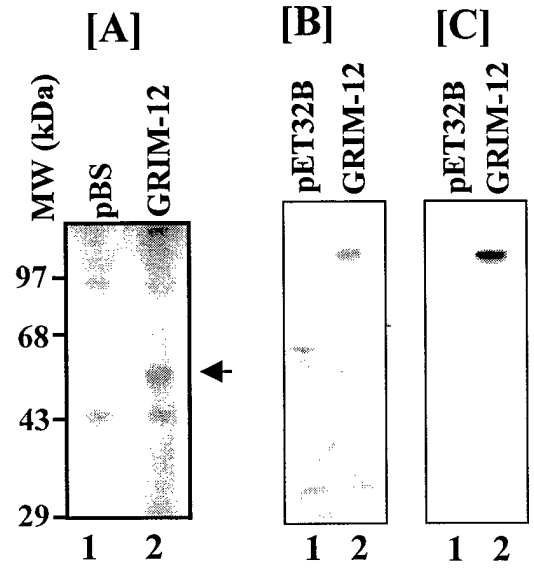


Figure 2

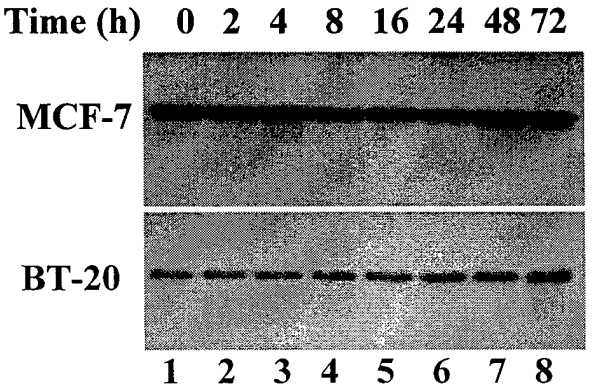


Figure 3

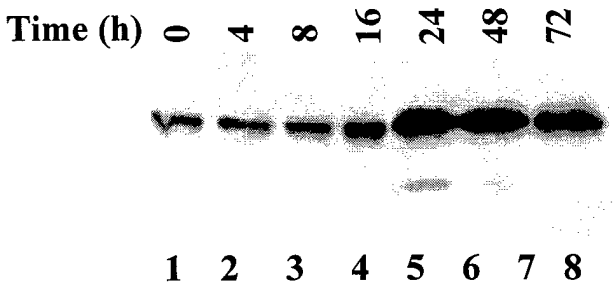


Figure 4

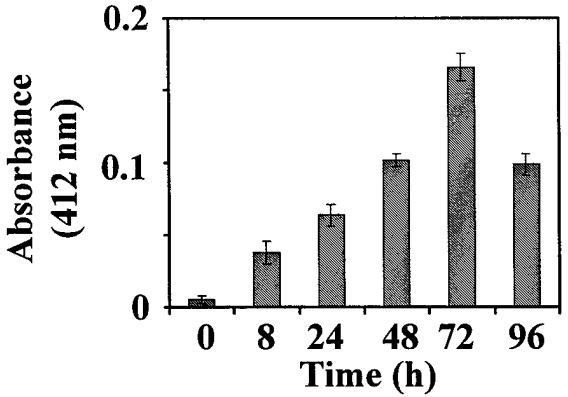


Figure 5

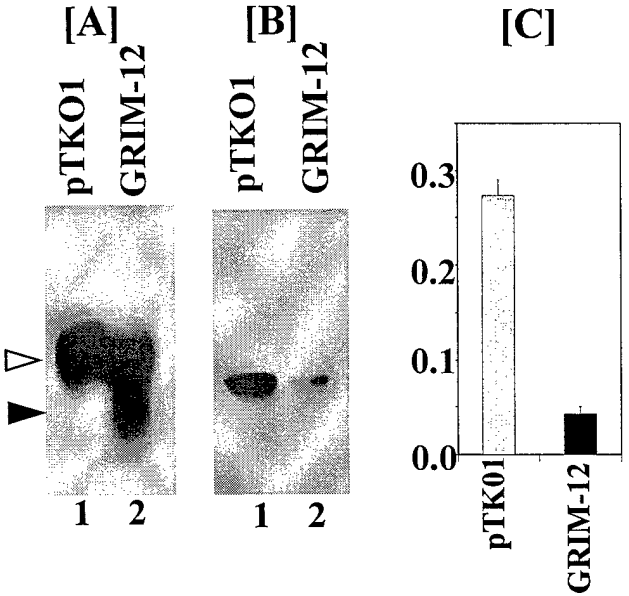


Figure 6

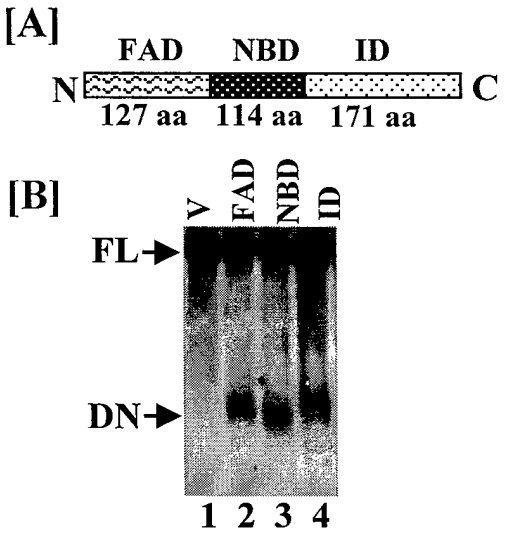
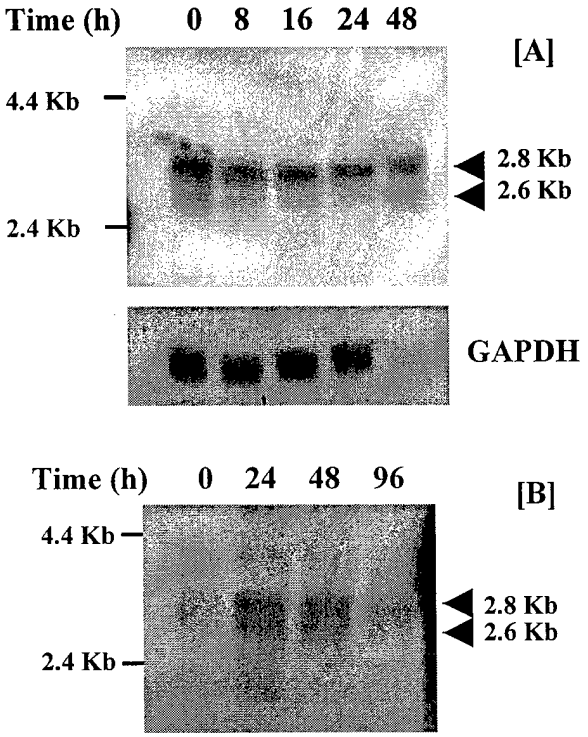


Figure 7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[A]
4.4 Kb
2.4 Kb
2.8 Kb
2.6 Kb
GAPDH
Time (h) 0 24 48 96
[B]
4.4 Kb
2.4 Kb
2.8 Kb
2.6 Kb

Figure 8